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## **REMARKS/ARGUMENTS**

In response to the Office Action of June 3, 2004, Applicants request re-examination and re-consideration of this application for patent pursuant to 35 USC 132.

### **Claim Status/Support for Claim Amendments**

Claim 13 has been cancelled. Claims 1-12 and 14-20 have been withdrawn as the result of the previous restriction requirement. In view of the Examiner's restriction requirement, applicants retain the right to present claims 1-12 and 14-20 in a divisional application. Claims 21-23 have been added. Claims 1-12 and 14-23 are pending in the instant application.

The addition of claims 21-23 does not add new matter to the specification. The subject matter and terms of new claims 21-23 can be found in the originally filed claims. The subject matter of claim 21 corresponds to the subject matter of cancelled claim 13. Support for claim 21 can also be found in the originally filed specification at page 1, lines 6-10; page 9, lines 14-19 and page 11, line 6 to page 13, line 11. The term "Schwann cell autoantibody" is found at page 7, lines 1-7 and page 8, line 21 to page 9, line 4 of the originally filed specification. The term "immunologically detectable fragment" is found at page 16, lines 1-15 of the originally filed specification. Support for claim 22

can found at page 7, lines 1-14 of the originally filed specification. Support for claim 23 can be found at page 18, lines 10-14 of the originally filed specification.

#### Objection to the Specification

The Examiner has objected to the specification as allegedly failing to provide proper antecedent basis for the claimed subject matter (37 CFR 1.75(d)(1) and MPEP 608.01(o)). The Examiner asserts that the specification fails to teach the detection of anti-GFAP using IgG.

Applicants respectfully disagree with the Examiner's assertion. Claim 13, which represents the claimed subject matter under examination, has been cancelled. The subject matter of new claim 21 corresponds with the subject matter of cancelled claim 13. The analyzing step of new claim 21 is not limited as to how the analyzing is carried out, however, detection of anti-GFAP using IgG is taught by the instant specification. Page 11, lines 20-23 state that samples with high autoantibody signals in SELDI-TOF-MS were found to contain anti-GFAP autoantibodies in Western blots, but the sensitivity of SELDI exceeds that of Western blots. A Western blot is a well-known technique which utilizes antibodies to detect a protein of interest (anti-GFAP, in the instant case).

Regardless of these teachings, extensive discussion of such techniques is not required in the specification considering that

techniques for the detection of proteins (in this instance the protein is anti-GFAP autoantibody) using immunoglobulins of the G isotype are well-known and in common practice, since it has been established that a patent need not teach and preferably omits what is well-known in the art (MPEP 2164.01).

Thus, considering the remarks presented in the above paragraphs, Applicants respectfully submit that the instant specification does provide proper antecedent basis for the claimed subject matter and request that this objection to the specification now be withdrawn.

**Rejections under 35 USC 112, second paragraph**

Claim 13, as originally presented, stands rejected under 35 USC 112, second paragraph as being indefinite for allegedly failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

The Examiner alleges that claim 13 is indefinite because it lacks a correlation between the detection of GFAP antibodies and fragments thereof and the diagnosis of prediabetes. The recitation of "a diagnostically effective amount" is unclear since this amount has not been defined, thus the boundaries of the claim are not discernible. The recitation of "prediabetes" is confusing since "prediabetes" is not a disorder per se. The claim is also confusing since it is unclear if it is a method of diagnosing type 1

diabetes, or if it is a method of screening for prediabetes. The recitation of "anti-GFAP IgG useful as a predictive marker of Type 1 diabetes" does not allow the metes and bounds of the claim to be ascertained. If autoantibodies to GFAP are useful as a predictive marker of Type 1 diabetes, will their detection or presence be indicative of prediabetes, regardless of clinical form (Type 1 or Type 2).

Applicants respectfully disagree with all of the Examiner's assertions. Claim 13 has been cancelled. New claim 21 corresponds to the subject matter of cancelled claim 13. Claim 21 clearly indicates that the presence of at least one Schwann cell autoantibody or immunologically detectable fragment thereof is diagnostic for pre-Type 1 diabetes. Thus, a clear correlation exists between the presence of the autoantibody and the diagnosis of pre-Type 1 diabetes, i.e. the presence of the autoantibody identifies pre-Type 1 diabetes. The phrase "diagnostically effective amount" is not recited in claim 21.

Applicants respectfully assert, contrary to the Examiner's opinion, that "pre-diabetes" (pre-Type 1 diabetes) is a recognized disorder. Pre-Type 1 diabetes is known in the art as a slowly progressive process wherein the autoreactive T-cells first surround the islets and eventually invade the interior of the islet resulting in the progressive loss of functioning  $\beta$  cells; see page 2, lines 1-5, 12-17 and page 13, lines 17-19 of the instant

specification. Furthermore, Naserke et al. (Journal of Clinical Endocrinology and Metabolism 84(4):1239-1243 1999) use the term "pre-diabetes" in a 1999 article discussing insulin autoantibodies which are early sensitive markers of pre-diabetes. Thus, demonstrating that the term "pre-diabetes" was commonly used in the art before the filing date of the instant application and therefore would not be confusing to those of skill in the art.

The method of claim 21 clearly relates to the diagnosis of pre-Type 1 diabetes when the presence of a Schwann cell autoantibody (or immunologically detectable fragment thereof) is detected within a sample of bodily fluid. Applicants were the first to recognize that if a diagnostic assay and method could be developed based on the detection of these autoantibodies which are produced during the pre-diabetes stage; it may be possible to improve treatment options and/or delay the clinical onset of diabetic symptoms prior to  $\beta$  cell destruction.

The phrase "anti-GFAP IgG useful as a predictive marker of Type 1 diabetes" is not recited in claim 21.

Applicants specifically claim a method for diagnosing pre-Type 1 diabetes, and thus contend that the question of whether the method can be applied to Type-2 diabetes is irrelevant to the claims currently presented for examination.

Accordingly, Applicants have now clarified the metes and bounds of the claims and respectfully request that the above-

discussed rejections under 35 USC 112, second paragraph be withdrawn.

**Rejection under 35 USC 112, first paragraph**

Claim 13, as originally presented, stands rejected under 35 USC 112, first paragraph for allegedly failing to comply with the enablement requirement. The Examiner asserts that the claim contains subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention. The claim is directed to a method for prediabetes screening by detecting autoantibodies to glial fibrillary acidic protein (GFAP) and such a method is not enabled by the specification as originally filed.

Applicants respectfully disagree with the Examiner's position. First, it is well established that claims are interpreted in light of the specification with regard to questions of enablement. Thus, in the instant case, the specification should enable the evaluation of body fluid samples for the presence of at least one Schwann cell autoantibody or immunologically detectable fragment thereof wherein the presence of such Schwann cell autoantibodies or fragments thereof identifies patients with pre-Type 1 diabetes who are at risk for development of Type 1 diabetes. Figure 2 shows data obtained from the evaluation of samples from 4 week old NOD male

and female mice. The presence of a Schwann cell autoantibody (anti-GFAP) was identified in female mice but not in male mice. Figure 3 shows similar data obtained from evaluation of 5 week old NOD mice. Female NOD mice develop Type 1 diabetes at a high rate while male NOD mice rarely develop the disease (page 7, lines 9 and 10 of the instant specification). Figures 4A-D show data obtained from evaluation of samples from patients; Figure 4B shows patients having recent onset Type 1 diabetes, Figure 4A shows patients having probable pre-Type 1 diabetes and Figures 4C and 4D show patients who show no signs of auto-immunity. The presence of a Schwann cell autoantibody (anti-GFAP) was identified in both patients having Type 1 diabetes (Figure 4B) and pre-Type 1 diabetes (Figure 4A) but was not found in patients showing no signs of auto-immunity (Figures 4C and 4D). Thus, the instant specification as originally filed clearly demonstrates the identification of a Schwann cell autoantibody in body fluid samples (of both NOD mice and human patients) which is associated with pre-Type 1 diabetes. Such an association would be recognized by a person of skill in the art when presented with the data shown in the instant specification.

Furthermore, all that is necessary is that one skilled in the art be able to practice the claimed invention, given the level of knowledge and skill in the art (MPEP 2164.08). Applicants assert that the skill in the art is high and it is obvious that no undue

experimentation would be required for a skilled artisan to follow any of the mass spectrometric (SELDI, as shown in Figure 1) or immunologic assay protocols (page 16 to page 18, line 14) presented in the instant specification in order to practice the claimed method. Thus, Applicants assert that one of skill in the art would be able to carry out the claimed method in order to identify patients having pre-Type 1 diabetes, since the specification establishes an association between Schwann cell autoantibodies and pre-Type 1 diabetes and the protocols used are well-known.

The Examiner asserts that the specification fails to teach one skilled in the art whether autoantibodies to GFAP are positively correlated with prediabetes or with diabetes. The evidence presented is not convincing because it fails to take into consideration the required criteria for a diagnostic assay.

The Examiner contends that a number of characteristics need to be considered in the development of any suitable diagnostic assay. These characteristics, upon which the Examiner appears to rely, are found in Strongin (1993, "Sensitivity, Specificity, and Predictive Value of Diagnostic Tests: Definitions and Clinical Applications", in Laboratory Diagnosis of Viral Infections, Lennette, editor, Marcel Dekker, Inc. New York, pages 211-219), which is allegedly relevant to the instant invention. The Strongin reference describes a set of statistical characteristics that a clinician can apply to confirm or exclude the diagnosis of a



disease. Each of the diagnostic procedures possesses a set of characteristics that determine how close the procedure in question compares to an "ideal" test, that is, one with 100% specificity and sensitivity, which as the text states is extremely uncommon.

The Examiner appears to believe that since the specification allegedly lacks any of the statistical characteristics stated in the Strongin reference, it would require undue experimentation for one skilled in the art to make and use the invention.

Applicants assert that the Strongin reference does not control the question of enablement. The guidelines for a "test of enablement" indicate that if a statement of utility in the specification contains within it a connotation of how to use, 35 USC 112 is satisfied. The instant application discloses a method for diagnosing pre-Type 1 diabetes through the detection of Schwann cell autoantibodies; markers which are produced during the pre-diabetes stage. The data presented in Figures 2-4 clearly show a positive correlation between Schwann cell autoantibodies (anti-GFAP) and pre-diabetes or diabetes. Such autoantibodies have not previously been shown to be associated with pre-Type 1 diabetes. When a marker is discovered to be associated with a disease state, it's potential for diagnostics and/or therapeutics is immediately recognized, even if the involvement of the marker in disease pathology is unknown. One of skill in the art would be familiar with this practice since it has been known in the art since at

least 1992. See attached abstract of Gunnensen et al. (Proceedings of the National Academy of Science USA 89(24):11949-11953 1992) in which the detection of glutamine synthetase in the cerebrospinal fluid of Alzheimer's disease patients lead to the suggestion of glutamine synthetase as a potential diagnostic marker for Alzheimer's disease. Thus, when one of skill in the art reviews the instant specification and observes the data indicating an association between Schwann cell autoantibodies and pre-Type 1 diabetes; one of skill in the art would connect Schwann cell autoantibodies with potential diagnostics and/or therapeutics for pre-Type 1 diabetes. Thus, compliance with the "required" criteria for a diagnostic assay according to Strongin is not necessary to show that the instant invention is enabled. When subjected to the "test for enablement" the Examiner's argument is not sufficient to support the enablement rejection; since the association of the autoantibody with pre-Type 1 diabetes carries with it a connotation of use for diagnostics.

The Examiner alleges that the specification lacks any teaching of how the diagnostic tests were performed. Applicants respectfully disagree with this statement as Figure 1 shows a schematic which clearly outlines the protocol for the diagnostic tests. Further, page 16 to page 18, line 14 discusses immunological assays which may be used to carry out the instant invention.

The Examiner also alleges that the specification does not

provide any information regarding the patients from which the samples were taken. Applicants respectfully disagree with this statement. The health status of the patient with regard to diabetes is the only information necessary to carry out the claimed method and the instant specification clearly provides this information. Page 12, line 21 to page 13, line 7 of the instant specification discloses the patients used to obtain the data presented in Figure 4. For example, patients of Figure 4B had recent onset Type 1 diabetes, patients of Figure 4A were autoantibody-positive first degree relatives with probable prediabetes and the patients of Figures 4C and 4D were relatives who showed no signs of autoimmunity.

Claims 21-23 have been added herein to clearly illustrate a method for detecting the presence of at least one Schwann cell autoantibody which is diagnostic for pre-Type 1 diabetes. The presence of Schwann cell autoantibodies in a patient identifies a patient in a pre-diabetic stage who is at risk for the development of clinical symptoms of Type 1 diabetes. The specification teaches using covalently GFAP-coupled chip arrays in combination with a SELDI-TOF-MS instrument for detection of the autoantibodies (page 11, lines 9-23 and Figure 1). Using similar techniques, samples with high autoantibody signals were found to contain GFAP autoantibodies in both NOD mice and humans (see page 12, lines 1-5; page 13, lines 3-7; Figures 2-4). As with conventional mass

spectrometry techniques, the presence of the autoantibody is determined by observation of a signal in the serum of a patient having recent onset of Type 1 diabetes; see Figure 4B, and relatives of the patient having probable pre-Type 1 diabetes; see Figure 4A, which may be quantified by comparison with control samples, as seen in Figures 4C and 4D, and discussed on page 12, line 21 to page 13, line 7. Intervention therapies are most effective before the development of insulinitis. Until the discovery made by the instant inventors there was no serum marker which could predict the development of diabetes before insulinitis is established. Invasive insulinitis develops in NOD mice at 10-12 weeks of age (see page 12, lines 6-20). It was found that in the case of NOD female mice, GFAP autoantibodies have a positive predictive power of about 90% at the age of 5 weeks, i.e. before insulinitis is established (see page 12, lines 14-16). Thus, it is likely that treatment administered at this stage will be effective for preventing further development of diabetes. As shown by the above arguments, the instant specification, contrary to the Examiner's opinion, does contain proper guidance to enable one of ordinary skill in the art to practice the claimed method for diagnosing pre-Type 1 diabetes without any undue experimentation.

The Examiner alleges that the prior art is silent on any correlation between autoantibodies to GFAP and diabetes or prediabetes.

It has been established that the mere fact that something has not previously been done clearly is not, in itself, a sufficient basis for rejecting all applications purporting to disclose how to do it. Although the prior art does not specifically recognize that GFAP autoantibodies are correlated with pre-diabetes, it does recognize that autoimmunity is involved with Type 1 diabetes; for example, see the attached abstract of Colman et al. (Pediatric Diabetes 1(4):193-198 2000) which discusses a screening for children having subclinical Type 1 diabetes (pre-Type 1 diabetes). Colman et al. states specifically that Type 1 diabetes is recognized to have a subclinical phase during which anti-islet antibodies can be detected. Thus, one of skill in the art would not be completely surprised by the discovery that other autoantibodies to pancreatic tissues are involved in the pathogenesis of pre-Type 1 diabetes.

Additionally, it was also known in the art that neurons, (including Schwann cells) are damaged in diabetes; for example, see the attached abstract of Cameron et al. (Diabetes Research and Clinical Practice 45(2-3):137-146 1999) who report on the damage to Schwann cells caused by elevated reactive oxygen species (ROS) in diabetes. Thus, one of skill in the art would also not be completely surprised by the discovery that autoantibodies to Schwann cells are involved in the pathogenesis of Type 1 diabetes.

The Examiner further asserts that the art of record teaches

away from extrapolating data in NOD mice to humans; citing Atkinson et al. (Nature Medicine 5(6):601-604 1999) as a reference. From this article, the Examiner concludes that the teaching that because autoantibodies to GFAP are found in both NOD mice and humans, it is evidence for prediabetes is not convincing for two reasons: (1) autoantibodies to GFAP have not been definitely proven to be diagnostic of prediabetes in NOD mice, and (2) data from NOD mice cannot always be extrapolated to humans.

Applicants respectfully disagree with the Examiner's interpretation of the Atkinson reference. Atkinson does not discourage investigations using NOD mice nor does Atkinson teach away from extrapolating data obtained from these investigations to humans. Atkinson states the following at page 601, first column on the left:

"Today, when a candidate autoantigen undergoes evaluation, the effect of a cytokine is tested, or a preventative intervention is assessed, NOD mice are often considered as good as it gets, short of a study in humans -so much so that other animal models are not always tested **nor are important distinctions with the NOD model considered before extrapolations to humans are made**"

Thus, contrary to the Examiner's interpretation, Atkinson actually encourages extrapolations of data from NOD mice investigations to humans.

Additionally, Atkinson states at page 604, first full

paragraph, "At a minimum, investigations of NOD mice have enhanced our appreciation of the etiologic complexity of Type 1 diabetes in humans and provided an example of how promising the results in the animal model can be translated into human clinical trials".

This statement of Atkinson also supports extrapolation of data from NOD mice investigations to humans.

Much of the prior art in diabetes research utilizes NOD mice due to the difficulty inherent in studying diabetes in humans. Animal models of disease offer many advantages. Diabetic animals can be biopsied and autopsied. They can be bred to study and manipulate inheritance. See Rossini et al. (Clinical Immunology and Immunopathology 74(1):2-9 1995) Rossini et al. additionally report that "Few facets of the immune systems of these have escaped study, and the information gathered has led directly to clinical trials in children". (see page 3, first full paragraph).

Thus, Applicants respectfully submit that the NOD mouse is an art recognized model of human Type 1 diabetes and that the extrapolation of data derived from studies using these NOD mice is common practice.

In conclusion, Applicants claim that the presence of at least one Schwann cell autoantibody or immunologically detectable fragments thereof is diagnostic for pre-Type 1 diabetes; a statement which is enabled by the data presented in the specification as originally filed. Applicants assert that one of

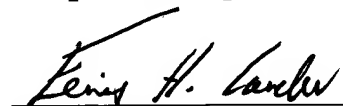
ordinary skill in the art when reviewing the instant specification would recognize how to use the claimed method for the diagnosis of pre-Type 1 diabetes. Thus, Applicants respectfully request that this rejection under 35 USC 112, first paragraph now be withdrawn.



CONCLUSION

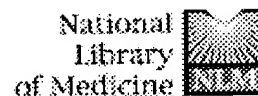
In light of the foregoing remarks and amendments to the claims, it is respectfully submitted that the Examiner will now find the claims of the application allowable. Favorable reconsideration of the application is courteously requested.

Respectfully submitted,

  
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## Screening for preclinical type 1 diabetes in a discrete population with an apparent increased disease incidence.

Colman PG, McNair P, King J, Caudwell J, Jankulovski C, Tait BD, Honeyman MC, Harrison LC.

Department of Diabetes and Endocrinology, Walter and Eliza Hall Institute of Medical Research, Royal Melbourne Hospital, Victoria, Australia. peter.colman@mh.org.au

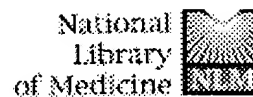
Environmental agents are proposed to play a role in triggering or exacerbating pancreatic islet autoimmunity in people genetically predisposed to type 1 diabetes. However, with few exceptions, these agents remain enigmatic. Clues to environmental agents may be found by investigating population/geographic clusters or 'hotspots' of high disease incidence. We were alerted to a small community where the incidence of type 1 diabetes appeared to be five-fold higher than expected. Because type 1 diabetes is now recognized to have a subclinical phase during which anti-islet antibodies can be detected, we aimed to identify and characterize a reservoir of children with subclinical disease in this community. Venous blood samples were collected from 1906/2347 (81%) local school children during one week. Islet cell antibodies (ICAs) were detected in 122 (6.4%) children, 18 (0.9%) being high titer ( $\geq 20$  Juvenile Diabetes Foundation units (JDFu)). On retest, 15 months later, the majority of low titer ICAs were undetectable, whereas high-titer ICAs persisted. The latter were found in two distinct age-related, ethnically similar groups. The younger group, aged 6-9 yr, had antibodies to insulin (IAAs), glutamic acid decarboxylase (GAD) and tyrosine phosphatase IA2 in addition to ICA, human leukocyte antigen (HLA) genes associated with susceptibility to type 1 diabetes, and lower first-phase insulin responses (FPIRs) to intravenous glucose. The older group, aged 13-16 yr, the age cohort of the index clinical cases, had few antibodies other than ICA, non-susceptibility HLA genes and normal FPIRs. During follow-up, three children, all from the younger group with multiple antibodies and FPIRs less than the first percentile, developed diabetes 4, 6 and 7 yr after screening. The finding of two age groups of subclinical disease suggests that if environmental agents triggered islet autoimmunity they did not act constantly on the community. Furthermore, the absence of multiple autoantibodies and/or HLA susceptibility genes in the older group, the source of index clinical cases, implies they are a residual subgroup with slow or absent progressive beta-cell destruction. This study illustrates that the natural history of type 1 diabetes may be elucidated by analyzing age-related subclinical disease in the general population.

PMID: 15016215 [PubMed]

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[PubMed Central](#)**Immunoglobulin G insulin autoantibodies in BABYDIAB offspring appear postnatally: sensitive early detection using a protein A/G-based radiobinding assay.****Naserke HE, Bonifacio E, Ziegler AG.**

Institute of Diabetes Research, Munich, Germany.

Insulin autoantibodies (IAA) are early sensitive markers of pre-diabetes in the young. The aim of this study was to assess whether, using IgG-specific measurement with a protein A/G assay, IAA are already present at birth, and whether this assay is suitable for early autoantibody screening. Cord blood and follow-up samples from offspring of parents with type 1 diabetes included in the BABYDIAB study were analyzed. Although insulin antibodies in cord blood from children of mothers with type 1 diabetes were readily detected and correlated well with levels in the maternal circulation, no insulin binding was detected in 247 cord blood samples from children of father probands. IgG IAA were detected at 2 yr in all 21 children who had multiple islet autoantibodies or who later developed type 1 diabetes, but were confirmed in only 6 of 58 with IAA by the conventional IAA assay in the absence of other islet autoantibodies. False positive IAAs in the conventional assay were often attributable to hemolysis. Hemolysis did not affect protein A/G IAA measurement, and results in whole capillary blood samples were comparable to those in corresponding serum samples ( $r^2 = 0.99$ ). These data show that IgG IAA appear early and after birth, and that the protein A/G IAA assay is sufficiently sensitive for early screening. The specificity of this assay requires further evaluation.

PMID: 10199761 [PubMed - indexed for MEDLINE]

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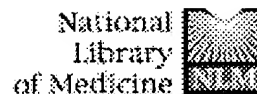
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## Effects of antioxidants on nerve and vascular dysfunction in experimental diabetes.

Cameron NE, Cotter MA.

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Reactive oxygen species (ROS) are elevated by metabolic changes in diabetes, including autoxidation and increased advanced glycation. Endogenous protection by the glutathione redox cycle is also compromised by the competing NADPH requirement of elevated polyol pathway flux. Antioxidant treatment strategies prevent or reverse nerve conduction velocity (NCV) deficits in diabetic rats. These include lipophilic scavengers such as butylated hydroxytoluene, probucol and vitamin E, more hydrophilic agents like alpha-lipoic acid and acetyl cysteine, and transition metal chelators that inhibit autoxidation. In the long-term, elevated ROS cause cumulative damage to neurons and Schwann cells, however, they also have a deleterious effect on nerve blood flow in the short term. This causes endoneurial hypoxia, which is responsible for early NCV deficits. Antioxidant treatment corrects the blood flow deficit and promotes normal endoneurial oxygenation. ROS cause antioxidant-preventable vascular endothelium abnormalities, neutralizing nitric oxide mediated vasodilation and increasing reactivity to vasoconstrictors. Unsaturated fatty acids are a major target for ROS and essential fatty acid metabolism is impaired by diabetes. Gamma-linolenic acid stimulates vasodilator prostanoid production, and there are marked synergistic interactions between gamma-linolenic acid and antioxidants. This has encouraged the development of novel drugs such as ascorbyl-gamma-linolenic acid and gamma-linolenic acid-lipoic acid with enhanced therapeutic potential.

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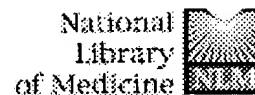
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in PubMed Central**Detection of glutamine synthetase in the cerebrospinal fluid of Alzheimer diseased patients: a potential diagnostic biochemical marker.****Gunnerson D, Haley B.**

Department of Biochemistry, College of Pharmacy, University of Kentucky, Lexington 40536-0084.

In this report, 8- and 2-azidoadenosine 5'-[gamma-32P]triphosphate were used to examine cerebrospinal fluid (CSF) samples for the presence of an ATP binding protein unique to individuals with Alzheimer disease (AD). A 42-kDa ATP binding protein was found in the CSF of AD patients that is not observed in CSF from normal patients or other neurological controls. The photolabeling is saturated with 30 microM 2-azidoadenosine 5'-[gamma-32P]triphosphate. Photoinsertion can be totally prevented by the addition of 25 microM ATP. Photoinsertion of 2-azidoadenosine 5'-triphosphate into the protein is only weakly protected by other nucleotides such as ADP and GTP, indicating that this is a specific ATP binding protein. A total of 83 CSF samples were examined in a blind manner. The 42-kDa protein was detected in 38 of 39 AD CSF samples and in only 1 of 44 control samples. This protein was identified as glutamine synthetase [GS; glutamate-ammonia ligase; L-glutamate:ammonia ligase (ADP-forming), EC 6.3.1.2] based on similar nucleotide binding properties, comigration on two-dimensional gels, reaction with a polyclonal anti-GS antibody, and the presence of significant GS enzyme activity in AD CSF. In brain, GS plays a key role in elimination of free ammonia and also converts the neurotransmitter and excitotoxic amino acid glutamate to glutamine, which is not neurotoxic. The involvement of GS, if any, in the onset of AD is unknown. However, the presence of GS in the CSF of terminal AD patients suggests that this enzyme may be a useful diagnostic marker and that further study is warranted to determine any possible role for glutamate metabolism in the pathology of AD.

PMID: 1361232 [PubMed - indexed for MEDLINE]

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## COMMENTARY

Of the two well-known rodent models of human type 1 diabetes (the non-obese diabetic mouse and the BioBreeding rat) the mouse has become the model of choice. Here the authors re-examine the value of this mouse model as a tool for understanding human diabetes and for testing potential therapies, reviewing both strengths and weaknesses.

## The NOD mouse model of type 1 diabetes: As good as it gets?

Diabetes mellitus in humans is a genetically and clinically heterogeneous group of glucose intolerance syndromes. Type 2 diabetes (also called non-insulin-dependent diabetes mellitus) is the more prevalent clinical form, in which obesity associated with progressively more severe insulin resistance are common predictors of the prediabetic state. Type 1 diabetes (also called insulin-dependent diabetes mellitus, or juvenile diabetes), in contrast, usually has an autoimmune T cell-mediated etiology in which the prediabetic state is characterized by development of autoantibodies against certain proteins expressed by  $\beta$  cells, including insulin. Two rodent models that spontaneously develop type 1 diabetes, the NOD (non-obese diabetic) mouse and the BB (BioBreeding) rat, have allowed detailed exploration of the dysregulated communication between cells of the innate and acquired immune system that underlie the generation and release of pancreatic  $\beta$  cell-reactive T cells.

In the 19 years since the first report of the NOD mouse, this small rodent has eclipsed its 'bigger brother', the BB rat, as the favored model for investigations into the etiopathogenesis of autoimmune, T cell-mediated type 1 diabetes in humans. The reasons for the preferred popularity of the mouse model include a better-defined genome,<sup>1</sup> more monoclonal reagents for the analysis of immune system components and considerably lower maintenance costs. Today, when a candidate autoantigen undergoes evaluation, the effect of a cytokine is tested or a preventative intervention is assessed, NOD mice are often considered 'as good as it gets', short of a study in humans—so much so that other animal models are not always tested nor are important distinctions with the NOD model considered before extrapolations to humans are made.

Indeed, the introduction of NOD mice to diabetes

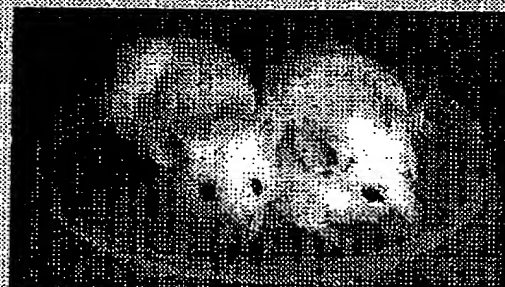
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research infused a large sense of optimism, with immunologists initially assuming that the NOD mouse would be a murine 'Rosetta stone' for quickly unraveling the secrets of the etiopathogenesis of type 1 diabetes in humans. This belief found early support through the observation that, as in humans, the major histocompatibility complex (MHC) of the NOD mouse (designated H2<sup>d</sup>) contributed the main component of susceptibility, and that the MHC class II I-A $\beta$  chain showed the same 'diabetogenic' amino-acid substitution found in the human DQ\*0302 allele associated with high risk for development of type 1 diabetes (a non-aspartic acid substitution at residue 57 in the  $\beta$ -chain). Rapid cloning of the effector T cell, sequencing of its T-cell receptor (TCR) genes and identification of cognate peptide(s) were predicted to follow. From there, identification of the target  $\beta$ -cell autoantigen and development of blocking peptides or tolerogenic regimens were to be relatively simple matters. Furthermore, it was assumed that identification of non-MHC

diabetogenic loci in the NOD mouse would allow for rapid identification of human homologs, thereby allowing accurate prediction of children at high genetic risk for developing type 1 diabetes. Finally, if the target autoantigen in mouse and human  $\beta$  cells were the same, the prevention of type 1 diabetes in the NOD mouse would rapidly be followed by comparable immunologic prediction and, hopefully, the eradication of this disease in humans.

Our understanding of the pathogenic mechanisms underlying type 1 diabetes development in NOD mice is now quite advanced<sup>1</sup>. However, this understanding has been accompanied by the realization that when this mouse is used as a surrogate for humans, genus-specific differences that restrict their interpretation are unavoidable. In addition to certain NOD strain-

**Origin of NOD mice:** The NOD mouse (depicted at left) arose from outbred 'Swiss' mice in the course of a selection experiment in Japan in which brother  $\times$  sister matings were being used to produce a strain in which all mice developed cataracts. At an early generation of inbreeding, mice without cataracts, but with elevated fasting blood glucose (a clinical sign of pre-diabetes) were noted. These were selectively bred in the hope of creating a mouse model for spontaneous diabetes development. The end result of the selection was the inbred NON strain depicted on the right, a model of pre-type 2 diabetes. The NOD strain (left) derived from a co-selected control line exhibiting normal fasting blood glucose levels, the first case of spontaneous autoimmune type 1 diabetes occurred unexpectedly in this 'homoglycaemic' control line at the twentieth generation of sibling mating. When these two related strains are intercrossed to elucidate the chromosomal locations of diabetes susceptibility genes, homozygous expression of the NOD MHC alleles is essential for disease development, in contradistinction to the genetics



of human type 1 diabetes, in which heterozygosity for MHC genes is common. When the chromosomal locations of non-MHC genes are identified, the NOD parental strain, as expected, contributes most of the genetic susceptibility. However, some susceptibility also derives from the NON strain genome. This illustrates the complexity of diabetes genetics in an outbred human population, wherein overlapping susceptibility for type 1 and type 2 diabetes may be inherited in some individuals.



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**Table The 'A-to-Z' of diabetes prevention in the NOD mouse. Therapies include those that either suppress T-cell functions or stimulate the immune system to achieve a more normal immunoregulatory communication between antigen-presenting cells and T cells.**

Androgen	Essential fatty acid-deficient diets	Interleukin-2	Overcrowding
Anesthesia	FK506	Interleukin-2 receptor fusion toxin (DAB480-IL-2)	Pancreatectomy
Azathioprine	Galium nitrate	Interleukin-3	Pentoxifylline
Anti-B7-1	Glucose (neonatal)	Interleukin-4	Pertussis toxin
Bacille Calmette Gue'rin (BCG)	Glutamic acid decarboxylase	Interleukin-10	Poly [I:C]
Baculofin	-intrapertitoneal, intrathymic, intravenous, oral	Interleukin-12 antagonist	Pregestimil diet
$\beta$ -1,6;1,3-D-glucan	Glutamic acid decarboxylase peptides	Islet cells-intrathymic	Probiolact
Anti- $\beta$ 7 integrin	-intrapertitoneal, intrathymic, intravenous, oral	Lactate dehydrogenase virus (LDH)	Rampamycin
Blocking peptide of MHC class II	Gonadectomy	Lactobacillus casei	Reg protein
Bone marrow transplantation	Heat shock protein 65	Lazaroid	Rolipram
Castration	Heat shock protein peptide (p277)	Linomide	Saline (repeated injection)
Anti-CD3	Anti-ICAM-1	Lithium chloride	Semi-purified diet (AIN-76)
Anti-CD4	Immobilization	Anti-LFA-1	Silica
Anti-CD8	Immunoglobulin (IgG2a)	Anti-L-selectin	Sodium fusidate
Anti-CD28	Anti-integrin alpha 4	Lymphocyte choriomeningitis virus (LCMV)	Somatostatin
Cholera toxin-B subunit	Inomide	Anti-lymphocyte serum/lymphotoxin	Non-specific pathogen free conditions
Cold exposure	Insulin	Lymphocyte vaccination	Streptococcal enterotoxins (SEA)
Anti-complement receptor	-intrapertitoneal, oral, subcutaneous, nasal	LZ8	Superantigens
Complete Freund's adjuvant	Insulin B chain/B chain amino acids 9-23	MDL 29311	Superoxide dismutase-desferrioxamine
Anti-CTLA-4	-Intrapertitoneal, oral, subcutaneous, nasal	Melatonin	TGF- $\beta$
Cyclosporin	Insulin-metabolically inactive	Anti-MHC class I	Anti-T-cell receptor
Cyclosporin A	Insulin-like growth factor I	Anti-MHC class II	Anti-thy-1
Dapsone (4,4'-diaminodiphenyl sulfone)	Interferon- $\alpha$	Mixed allogeneic chimerism	Thymectomy (neonatal)
Deflazacort	Anti-interferon- $\gamma$	Monosodium glutamate	T-lymphocyte clones
Dendritic cells from pancreatic lymph node	Interferon- $\gamma$ receptor	Murine hepatitis virus (MHV)	Tolbutamide
Deoxyspergualin	Interleukin-1	Mycobacterium	Troglitazone
Diazoxide	Interleukin-1 receptor	Natural antibodies	Tumor necrosis factor- $\alpha$
1,25 dihydroxyl Vitamin D3		Nicotinamide	Tumor necrosis factor- $\beta$
Elevated temperature		Nutramigen	Vitamin E
Encephalomyocarditis virus (ECMV)		OK432	Anti-VLA-4
Escherichia coli extract			

specific characteristics that distinguish these mice from humans at risk for type 1 diabetes (such as deafness or the absence of C5 complement), important genus-specific features distinguish the murine diabetes as well (such as resistance to ketoacidosis or the absence of the murine homolog of HLA-DR molecules on antigen-presenting cells). Investigators have not always considered that because these mice are so highly inbred, they must be viewed as a single 'case study' in humans. Indeed, the combination of NOD strain-specific features as well as inherent differences between genera may explain why identification of non-MHC diabetogenic loci in mice have not generally been direct guide posts for the identification of homologous loci in outbred humans at risk for type 1 diabetes. As an example, the non-MHC locus (*IDDM2*, chromosome 11) that contributes to increased sibling risk in human studies is associated with a variable-number tandem repeat controlling expression of the closely-linked insulin gene. A susceptibility-conferring homolog has not yet been identified in the NOD mouse, most likely because the mouse genome, unlike the human genome, contains two unlinked insulin genes, both of which are expressed. Nevertheless, certain immunogenetic and immunopathogenic aspects of type 1 diabetes in this mouse 'case study', particularly the main pathogenic contributions made by MHC genes (*idd1* in NOD mice and *IDDM1* in humans), clearly justify thorough investigation into why MHC-associated deficiencies in immune function allow development of an autoreactive T-cell repertoire.

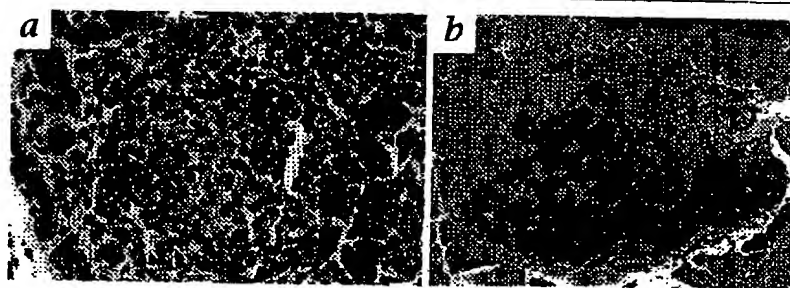
The etiology of type 1 diabetes in this model is both complex

and multifactorial<sup>1,3</sup>. Both CD4<sup>+</sup> and CD8<sup>+</sup> T cells constitute the effector arm, with underlying functional defects in bone marrow-derived antigen-presenting cells (APCs), including macrophages, dendritic cells and B lymphocytes, shown to be essential components in selection/activation of the autoimmune repertoire. Many CD4<sup>+</sup> and CD8<sup>+</sup> T-cell lines and clones with diabetogenic potencies against a variety of identified and unidentified antigens have been established from both islets and spleen<sup>2</sup>. If there is a single TCR clonotype distinguishing the 'primordial' diabetogenic T cell, its primacy has not yet been demonstrated. Destruction of  $\beta$  cells apparently entails both necrotic and apoptotic events in response to invasion of the islets by leukocytes (insulinitis)<sup>3</sup>. There are large numbers of leukocytes in the insulitic infiltrates of NOD mice, almost suggesting lymph node formation around islets. Insulinitis in a human acute-onset diabetic is very different from that in NOD islets (Fig.). One of the strain-specific peculiarities of NOD mice is the accumulation of many T cells in peripheral lymphoid organs, pancreas and submandibular salivary glands. This T-lymphocyte accumulation possibly reflects low IL-2 levels and the resistance of thymocytes and peripheral T cells to the induction of apoptosis.

Although they are important in improving our understanding of the cause(s) and pathogenesis of this disease, these immunologic features are also vital for this model to serve as a tool in identifying potential therapeutic modalities for the prevention of human type 1 diabetes. As of early 1999, more than 125 individual methods reporting the prevention or delay of

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**Fig. 1. Insulinitis in humans and NOD mouse pancreas.** *a*, Pancreas section with heavy leukocyte infiltration (insulitis) in a human islet. The donor died a few days after acute onset of type 1 diabetes. (photo courtesy of Massimo and Trucco, Department of Pediatrics, University of Pittsburgh). Hematoxylin and eosin stain. *b*, Pancreas of a prediabetic 12-week-old NOD/Lt mouse, showing the unusually heavy accumulation of leukocytes adjacent to and infiltrating the islets. The pancreatic  $\beta$  cells within the islet have been stained purple with aldehyde fuchsin.



type 1 diabetes in NOD mice have been identified (Table). These interventions can be grouped into two general categories: treatments that actually suppress T-cell function and treatments that modulate immune communication, often by actually stimulating certain immune functions. Although this list is long, we limited reference to studies monitoring spontaneous type 1 diabetes and excluded reports whose practical relevance to human disease is unclear. (For example, introduction of genetically-disrupted genes whose normal counterparts are required for (auto)antigen presentation, for T-cell effector functions, or for rearrangement of functional T-cell receptors.) The ease by which immunomodulation diverts the immune system in these mice is best understood by considering the effect of their exposure to extrinsic microbial pathogens. The inbreeding of NOD mice has genetically fixed a number of immunodeficiencies that, in aggregate, are reflected by impaired communication between APC and T cells. NOD macrophages have an impaired ability to activate regulatory T cells in an autologous mixed lymphocyte reaction. Comparable impairment in dendritic cell function has been seen in patients with recent-onset type 1 diabetes<sup>4</sup>. The NOD immunodeficiencies are partially correctable in a natural environment, in which a full range of microbial and viral antigens would be encountered. It is only when NOD mice are maintained in stringent specific pathogen-free conditions that full disease penetrance of the underlying genetic type 1 diabetes susceptibility will be seen in both sexes. Thus, the NOD model is one in which paradoxical immunostimulation effected by a variety of treatments ameliorates the weak communication between the innate and adaptive immune system components and thereby restores more normal control over autoreactive T cells. Unfortunately, in a genetically heterogeneous human population containing individuals at high risk for type 1 diabetes development, there is little evidence that many of them would have a comparable set of immune deficiencies that prove as malleable. At the same time, the observation that 'cleaning up' the extrinsic environment of the NOD mouse sets the stage for activation of autoimmune T cells raises the question of whether a hypersanitized rearing environment for human infants might predispose children with autoimmune-permissive HLA haplotypes to higher risk for eventual penetrance of autoimmune diseases.

In NOD mice, type 1 diabetes development is well-choreographed when all the relevant environmental factors (pathogen status, diet and so on) are held constant. Specific 'time windows' can be defined in which an immunomodulator can be either protective or destructive. In contrast, the natural history of type 1 diabetes in humans is such that the age of disease onset is extremely broad; symptoms occur at any time from the first years of life to well beyond 50 years of age. Although it is a potential limitation in comparing the natural

history of type 1 diabetes in NOD mice with humans, this factor can be turned into a strength through proper matching of therapeutic agents to the appropriate time for human intervention. Intervention studies in NOD mice can be designed in which therapeutic regimens are initiated at birth, at a presymptomatic stage before the occurrence of insulitis (that is, less than three weeks postpartum); before the onset of symptomatic disease (that is, four to eight weeks postpartum) at a time when considerable numbers of  $\beta$  cells are still intact; or at the diagnosis of type 1 diabetes, when  $\beta$ -cell damage has accumulated to the extent of overt hyperglycemia. However, studies analyzing therapeutic agents aimed at preventing type 1 diabetes in NOD mice must be carefully assessed for their functional as well as their practical applicability to therapeutic intervention in human disease. This has not always been considered. For example, agents used in NOD mice from birth (a time without  $\beta$ -cell destruction) may not be applicable to treatment of humans identified immediately before the onset of type 1 diabetes (when substantial  $\beta$ -cell destruction has occurred).

It is clear that the genus-unique and strain-specific aspects of diabetogenesis in NOD mice must be fully understood and appreciated if we are to know which therapeutic protocols are reasonable to extrapolate to humans and which are not. In addition, intervention protocols effective in preventing type 1 diabetes in NOD mice should be studied in as many animal models as are available. The rationale for implementing insulin prophylaxis therapy to prevent type 1 diabetes in humans was based on the observation that insulin treatment of both prediabetic NOD mice and BB rats retarded onset and reduced disease frequency. However, one may question whether another candidate  $\beta$ -cell autoantigen, glutamic acid decarboxylase (GAD), will provide similarly promising results. Isoforms of this enzyme are relatively easy to detect in human and rat  $\beta$  cells. In contrast, GAD protein is present at considerably lower concentrations in NOD islets and what little GAD67 isoform can be detected may not all derive from  $\beta$  cells. Nevertheless, NOD mice can be deviated from diabetes by early treatment with recombinant GAD protein or peptides. In contrast, GAD autoimmunity does not seem to be a factor in BB rats, emphasizing the point that many models should be evaluated before extrapolations to humans are attempted. With such an appreciation comes the realization that it is also essential to extend mechanistic studies in the NOD mouse to the rat models of spontaneous and induced type 1 diabetes. In addition to the lymphopenic BB/Wor diabetes-prone rat that develops type 1 diabetes spontaneously, type 1 diabetes can be induced in the non-lymphopenic BB/WOR diabetes-resistant substrain and in other strains by immunomodulation coupled with exposure to a parvovirus, Kilham rat virus<sup>5</sup>. Thus, it is the rat and not the mouse model that the investigator should first consider if the object is to an-

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alyze the potential of environmental viral pathogens to serve as diabetogenic triggers. New rat models spontaneously developing type 1 diabetes, such as the non-lymphopenic Komeda diabetes-prone (KDP) rat<sup>6</sup>, should provide additional pathogenic insights as they become available.

At a minimum, investigations of NOD mice have enhanced our appreciation of the etiologic complexity of type 1 diabetes in humans and provided an example of how promising results obtained in an animal model can be translated into human clinical trials. However, exploitation of the peculiarities of the NOD genome for clinical research is yet to be fully realized. Specifically, further investigation of NOD mice should advance our understanding of the genetic and pathophysiologic basis for other complex pathologies (such as thyroiditis, lupus, sialoadenitis, deafness and inflammatory bowel disease). The strain's robust breeding performance, its extensively characterized genome and the availability of type 1 diabetes-resistant MHC-congenic stocks render NOD mice ideal for outcross with other inbred strains carrying gene mutations for physical mapping/positional cloning analyses. Deficiencies in immunoregulation (such as dysfunctional NK cells or the absence of hemolytic complement) make NOD mice congenic for additional immunodeficiency genes (*scid* and *rug*) ideal hosts for carrying human cells, especially when they are further modified by the transgenic insertion of human genes and/or simultaneous elimination of select murine genes. This technology is providing stocks suitable for analysis of the growth, development and survival of human hematopoietic cells. The immunocompromised NOD mice should also prove useful for the study of human infectious diseases, including AIDS, filariasis and malaria. Gene targeting technology and the ability to produce tissue-specific knockouts of genes are allowing dissection of pathogenic pathways not easily amenable to study in humans.

In sum, criticisms of the inbred nature and controlled housing environment, the ability to change natural physiology through genetic manipulation and the relative ease for disease prevention have caused some to question whether the model is 'as good as it gets'. It is clear that the course of type 1 diabetes development in randomly breeding humans will not be as easily deviated as it is in highly inbred rodent models in which genetic risk is a constant such that interventions can be initiated at very early stages of pathogenesis. Thus, no investigator should assume that the available mouse and rat models spontaneously developing type 1 diabetes represent complete surrogates for humans. However, the fact that diabetes in these rodents develops spontaneously rather than in response to investigator-induced gene targetings allows acquisition of essential insights into the interactions between genes and environment that together trigger a complex disease.

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## ANIMAL MODELS OF HUMAN DISEASE

### Human Autoimmune Diabetes Mellitus: Lessons from BB Rats and NOD Mice—*Caveat Emptor*

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*It is a capital mistake to theorize before one has data. Insensibly, one begins to twist facts to suit theories, instead of theories to suit facts.*

Sherlock Holmes, *Scandal in Bohemia*

#### 1. AUTOIMMUNE DIABETES MELLITUS IN CHILDREN

Diabetes mellitus is not a single disease but rather a group of disorders all characterized by hyperglycemia. Most cases are classified as either insulin-dependent diabetes mellitus (IDDM or type 1) or non-insulin-dependent diabetes (NIDDM or type 2). Classical IDDM accounts for about 10% of the cases and is associated clinically with the rapid onset of polyuria, polydipsia, polyphagia, and weight loss in children and young adults. Pathophysiologically, the symptoms result from an absolute deficiency of insulin. This in turn leads to hyperglycemia and severe catabolism with release of free fatty acids from adipose tissue and hepatic overproduction of ketones. Unless given exogenous insulin, affected individuals succumb to progressive emaciation, ketoacidosis, and dehydration (1).

The absence of insulin in IDDM is due to selective destruction of pancreatic  $\beta$  cells. This destructive process was found by von Meyenberg to be associated with lymphocytic inflammation of the islets, a process that he termed insulinitis (2). His 1940 observation gave rise to the hypothesis that IDDM is an autoimmune disease. In the 1960s Gepts substantiated this concept in a study of 22 young persons with IDDM who had died within 6 months of onset; 15 had insulinitis (3).

The autoimmunity hypothesis received further support from the discovery of immunologic markers strongly associated with diabetes (4). Islet cell antibodies were found to predict the clinical course of disease and their disappearance correlated with loss of  $\beta$  cells. Other important autoantibodies found in patients with IDDM are directed against insulin and glutamic acid decarboxylase (GAD) (4). The discovery of permissive major histocompatibility complex (MHC) haplotypes also strongly suggested that IDDM requires immuno-

genetic susceptibility alleles. However, concordance for IDDM among identical twins is only 30–50%. This finding is consistent with the existence of genetic predisposition but at the same time implicates environmental modifiers that influence expression of the disease.

Clinical observations also support the autoimmune hypothesis of IDDM. The disease can appear intercurrently with other disorders thought to be autoimmune in origin. These include Addison's disease, vitiligo, and lymphocytic thyroiditis (4). A further convincing observation was made when a pancreas was transplanted from a nondiabetic donor to a diabetic identical twin. Despite the absence of transplantation barriers, inflammatory destruction of the transplanted  $\beta$  cells occurred, suggesting recurrence of a tissue specific autoimmune diathesis (5). In another case, IDDM occurred when bone marrow was transplanted from a diabetic donor to a nondiabetic HLA-identical sibling recipient (6). The evolution of the autoimmune hypothesis of IDDM eventually led to a trial of the immunosuppressive agent cyclosporin in children with the disorder; it was found to prolong endogenous insulin production (4). Unfortunately its toxicity was unacceptably high.

#### 2. ANIMAL MODELS OF IDDM

These data from human studies clearly indicate that knowledge of IDDM has advanced steadily, but the disease nonetheless remains refractory to cure or prevention. In part this is due to the difficulty inherent in studying IDDM in humans. The diseased organ is inaccessible; the genetics of those at risk cannot be manipulated; new therapies cannot readily be tested. To circumvent these impediments, investigators have sought to develop animal models of the disease (7). The strategy offers many advantages. Diabetic animals can be biopsied and autopsied. They can be bred to study and manipulate inheritance. Their genome can be altered. Provocation of IDDM constitutes a fruitful line

of exclusively animal research and therapies to prevent or reverse the disease can readily be tested.

The amount of information now known about diabetic animals is remarkably extensive. Two animal models in particular have provided data relevant to human IDDM: The BB rat and the NOD mouse. In each, the observation of insulinitis succeeded by selective  $\beta$  cell destruction and finally ketoacidosis propelled broad programs of research. Few facets of the immune systems of these rodents have escaped study, and the information gathered has led directly to clinical trials in children. The study of cyclosporin was engendered in this way, as were other ongoing studies of tolerance induction and disease prevention that will be described below.

Our goal is to review some of the major insights gained from the animal models of IDDM, but to do so mindful of the perspective of Sherlock Holmes cited above. To what extent is it, in fact, appropriate to theorize and extrapolate from rats and mice to children? Is there danger in twisting animal facts to generate human theories?

This question, as it applies to the animal models of disease, is an old one. Albert Renold and Eleazar Shafir were among the earliest proponents of the lessons that could be learned from the study of animal models of complex metabolic disorders (8). Both, however, em-

phasized repeatedly that lack of biological identity between animals and humans strictly limits the extent to which an animal disease can "model" a human one,

... unless the necessary caution and *wisdom* are applied in defining the limits of the representative parts of any given model, as well as precise limitations placed on the interpretation of any information obtained. (8, p. 3)

In the following synopses, we will try to point out what are the lessons and what are the caveats hidden among them. Tables 1-4 summarize much of the data in a format intended to facilitate interspecific comparisons of both rodent species and their human counterpart with IDDM. The tables highlight not only the similarities, but also the differences that are to be found.

### 3. DIABETES-PRONE AND DIABETES-RESISTANT BB RATS

Spontaneous hyperglycemia and ketoacidosis appeared in a colony of outbred Wistar rats at the Bio-Breeding Laboratories in the mid-1970s (9, 10). The affected animals were eventually inbred to create what is now the diabetes prone (DP) BB rat strain. Between 50 and 90 days of age, most DP-BB rats of both sexes develop pancreatic insulinitis that is rapidly followed by selective destruction of  $\beta$  cells. After the onset of hyperglycemia, the residual end-stage islets are small,

TABLE 1  
Clinical, Genetic, and Environmental Features of Autoimmune Diabetes in Three Species

	Human	NOD mouse	DP-BB rat	DR-BB rat
Clinical				
Onset	Spontaneous	Spontaneous	Spontaneous	Inducible
Ketosis	Severe	Mild	Severe	Severe
Genetics				
MHC	HLA-DQ $\alpha$ and $\beta$	Unique I-A absent I-E	RT1 <sup>a</sup>	RT1 <sup>a</sup>
Non-MHC	At least 2 loci	>15 loci	At least 2 loci; <i>lyp</i> causes lymphopenia	Not known
Gender	$\varnothing = \delta^a$	$\varnothing > \delta$	$\varnothing = \delta$	$\varnothing = \delta$
Environment				
Diet	Unknown; suspected association with BSA	Preventable by removal of protein or calorie restriction	Prevented by removal of casein or by restriction of essential fatty acids	Prevented by restriction of essential fatty acids
Habitat	No known effects	Prevented by high temperature; gnotobiosis increases incidence	Gnotobiosis does not prevent IDDM. Incidence higher and onset earlier in VAF colonies	Induction by depletion of RT6 <sup>+</sup> T cells does not occur in VAF animals
Virus prevention	Unknown	Sendai; LCMV; LDHV Pichinde; MHV; vaccinia	LCMV	Unknown
Virus induction/association	Mumps; rubella; coxsackie B4	Unknown; retroviruses present	Unknown	KRV

Note. MHC, major histocompatibility complex; HLA, human leukocyte antigen; LCMV, lymphocytic choriomeningitis virus; LDHV, lactate dehydrogenase virus; MHV, murine hepatitis virus; BSA, bovine serum albumin; VAF, viral antibody free; KRV, Kilham rat virus; IDDM, insulin-dependent diabetes mellitus.

<sup>a</sup> The male to female ratio in human IDDM reportedly varies from 0.3 to 2.1 when selected ethnic national subpopulations are considered, but worldwide incidence data suggest that there is no consistent excess of cases in either sex (16). A recent large-scale collaborative European study reported a slight excess in incidence among males ( $\delta/\varnothing = 1.06$ , confidence limits 0.99 to 1.14), but this excess was not statistically significant (17).

TABLE 2  
Immunological Characteristics of Autoimmune Diabetes in Three Species

	Human	NOD mouse	DP-BB rat	Induced IDDM in DR-BB rat
Islet morphology	Selective $\beta$ cell destruction, insulinitis (less with older age at onset)	Peri-insulinitis followed by insulinitis, selective $\beta$ cell destruction.	Selective $\beta$ cell destruction, insulinitis	Selective $\beta$ cell destruction, insulinitis
Associated autoimmune diseases	Thyroiditis; adrenalitis; vitiligo; PA; polyendocrine syndromes	Sialoadenitis, thyroiditis	Thyroiditis	Thyroiditis
Abnormalities of humoral immunity	Antibodies to islet cell surface and cytoplasm, GAD, insulin, carboxypeptidase H, and other islet antigens	Same	Same, but presence of insulin autoantibodies controversial	Unknown
Evidence for abnormal cellular immunity	Adoptive transfer of IDDM by bone marrow	Adoptive transfer by bone marrow, T cells, T cell lines, and clones	Lymphopenia; adoptive by bone marrow, T cells	Adoptive transfer by bone marrow, sorted T cells, thymocytes
Accelerators of disease	No proven accelerators of disease onset.	Cyclophosphamide accelerates onset	Poly (I:C) accelerates onset	Induced by cyclophosphamide, low-dose radiation, poly (I:C), and anti-RT6 mAb

Note. PA, pernicious anemia; GAD, glutamic acid decarboxylase; poly (I:C), polyinosinic polycytidylic acid; mAb, monoclonal antibody.

distorted, and comprised predominantly of non- $\beta$  cells. Early in the program of inbreeding, at the sixth generation, animals that failed to develop diabetes were selected to start a control line of nondiabetic BB rats. Now designated as diabetes-resistant (DR) BB rats, these coisogenic descendants of DP forbears have a cumulative incidence of spontaneous diabetes of <1%. All BB rats express the RT1<sup>u</sup> major MHC haplotype which appears to be required for the expression of IDDM.

DP-BB rats are severely lymphopenic, particularly with respect to the CD8<sup>+</sup> and RT6<sup>+</sup> phenotypes. They are consequently susceptible to infections, prone to B cell lymphomas, and able to reject allografts only poorly. The gene responsible for lymphopenia in DP rats, *lyp*, has been mapped to chromosome 4 (RNO4)

(11). The mechanism responsible for the T cell lymphopenia in the DP-BB rat and its exact role in the development of diabetes are unknown. Lymphopenia and diabetes susceptibility are inherited independently, and peripheral T cell lymphopenia clearly favors the expression of spontaneous hyperglycemia. Transfusions of spleen cells from MHC compatible donors prevent DP-BB rat IDDM if given early in life.

In addition to the pancreatic histopathology and lymphopenia, many other observations suggest a role for the immune system in the pathogenesis of spontaneous DP-BB rat diabetes (9, 10). Neonatal thymectomy and administration of antilymphocyte serum prevent the disease. Mitogen-activated spleen cells from acutely diabetic donors accelerate disease in young DP rats and transfer it to MHC-compatible recipients.

TABLE 3  
Immunosuppressive Therapy of Autoimmune Diabetes in Three Species

	Human	NOD mouse	DP-BB rat	Induced IDDM in the DR-BB rat
Generalized immunosuppression	Cyclosporin prolongs endogenous insulin production if given at onset	Cyclosporin, FK506 prevent diabetes	Cyclosporin, FK506, thymectomy, ALS, radiation prevent diabetes and thyroiditis	Cyclosporin prevents diabetes and thyroiditis
Selective immunosuppression with monoclonal antibodies	Unknown	Prevention by antibodies against CD3, the TcR, V $\beta$ 8, CD4, CD8, MHC Class I, MHC class II, CD45, IFN- $\gamma$ , IL-6, the IL-2 receptor	Prevention by antibodies against CD8, ASGM1, CD3	Prevention by anti-CD8

Note. TcR, T cell receptor; IFN, interferon; ASGM1, asialo-GM1; ALS, antilymphocyte serum.

TABLE 4  
Other Immunotherapies of Autoimmune Diabetes in Three Species

	Human	NOD mouse	DP-BB rat	Induced IDDM in the DR-BB rat
Cytokine therapy	Unknown	IL-1, IL-4, TNF $\alpha$ , IFN- $\gamma$ , IL-10 prevent diabetes	Unknown	Unknown
Immunostimulation				
Poly(I:C)	Unknown	Prevents diabetes	Accelerates onset	Induces diabetes
CFA	Unknown	Prevents diabetes	Prevents diabetes	Unknown
HSP65	Unknown	Prevents diabetes	Unknown	Unknown
BCG	Unknown	Prevents diabetes	Prevents diabetes	Unknown
Ciamezone	Unknown	Prevents diabetes	Does not prevent diabetes	Unknown
Peptide immunization	Unknown	HSP, insulin $\alpha$ and $\beta$ chains, glucagon, viral peptides	Unknown	Unknown
Miscellaneous				
Nicotinamide	Trial in progress	Prevents diabetes	Does not prevent diabetes	Unknown
Parenteral insulin	Trial in progress	Prevents diabetes even at doses that do not produce hypoglycemia	Prevents IDDM at doses that cause hypoglycemia	Prevents IDDM at doses that cause hypoglycemia
Oral insulin	Unknown	Prevents diabetes	Unknown	Unknown
Vitamins	Unknown	D, E prevent diabetes	Unknown	Unknown
Caramel food coloring	Unknown	Prevents diabetes	Unknown	Unknown
Androgen	Unknown	Prevents diabetes in $\varnothing$	Does not prevent diabetes	Unknown

Note. TNF, tumor necrosis factor; CFA, complete Freund's adjuvant; HSP, heat shock protein; BCG, Bacillus Calmette-Guerin.

Both anti-islet cell and anti-GAD autoantibodies occur in these animals. In contrast to humans with IDDM, anti-insulin antibodies appear to be absent (12), DP-BB rats also develop spontaneous lymphocytic thyroiditis but not clinical hypothyroidism.

Studies of diabetes prevention in the DP-BB rat have been complemented by studies of its induction in the coisogenic DR-BB strain (9, 10). DR-BB rats are not lymphopenic, but selective depletion of certain peripheral T cell subsets, those expressing the RT6 alloantigen, induces diabetes in these animals. Diabetes in DR rats can also be induced by low-dose irradiation, cyclophosphamide, viral infection, or administration of polyinosinic:polycytidylic acid (poly(I:C)), a synthetic double-stranded polyribonucleotide that elicits immune responses analogous to those observed during viral infection.

#### 4. NOD MICE

Makino and his colleagues reported a mouse model of spontaneous autoimmune diabetes in 1980 (7, 13, 14). These nonobese diabetic (NOD) mice develop insulinitis when 4 to 5 weeks old. By 7 months, 80% of female and 20% of male mice typically become diabetic. Ketoacidosis in affected animals is mild. Diabetic animals can survive for up to a month without exogenous insulin, but eventually insulin is required for survival. Morphologically "nests" of lymphocytes are observed jux-

tailed to the islets of Langerhans at  $\approx$ 5 weeks of age. As NOD mice mature, these mononuclear cells appear to migrate toward the islets, and by the time clinical hyperglycemia occurs, frank insulinitis is present.

As in humans and BB rats, additional lines of evidence suggest that NOD mouse diabetes is an autoimmune disorder. Splenocytes from adult NOD mice can adoptively transfer disease to MHC compatible, immunodeficient recipients. Both CD4<sup>+</sup> and CD8<sup>+</sup> lymphocytes appear to be required. Recently, cell lines have been obtained from the inflamed islets of affected NOD mice; these can induce diabetes in appropriate recipients (15).

A large number of both MHC and non-MHC genes have been linked to the development of NOD diabetes. The NOD expresses a unique I-A allele on chromosome 17 and lacks expression of I-E. At least 12 other non-MHC-linked genes have been implicated in the syndrome. As in the human and BB rat, anti-islet cell and anti-GAD autoantibodies are found in the NOD mouse. NOD mice also have sialoadenitis and thyroiditis.

The preponderance of diabetes in female compared with male NOD mice has prompted investigators to speculate that androgens exert a protective effect. Male castration does increase the frequency of diabetes. In the BB rat neither neonatal gonadectomy nor hypophysectomy changes the frequency of diabetes (9). In human IDDM both sexes are affected about equally (16, 17).



### 5. CAVEAT EMPTOR

Certain important differences between IDDM in humans and rodents are obvious from these descriptions. DP-BB rats are severely lymphopenic; humans and NOD mice are not. The risk of IDDM in NOD mice is far greater in females; this is not true of humans or BB rats. Ketoacidosis in humans and BB rats is more severe than in NOD mice. These obvious differences in disease phenotype clearly imply the existence of limits to the degree of analogy that can be ascribed to these three disease states.

The most fundamental limitation of the animal models is simply that we do not yet understand the precise cause of the disease in any of the species. We understand the elements the rodent diseases share with one another and with human IDDM only at a system level. The contribution of T lymphocytes to pathogenesis, the inflammatory lesion observed within the islets, the similar composition of end-stage islets, the importance of the MHC, the responsiveness to disruption of normal immune function—all of these are observations at the system level. These may be very useful observations, but it is essential to bear in mind that these similar systems may differ greatly at the cellular and molecular level. Knowledge of the system does not necessarily imply knowledge of the structural components of that system.

#### 5.1 The Problem of Genetic Drift

An obvious caveat with respect to interpreting data derived from animal models of IDDM is the heterogeneity that exists not only among species but also among the various rodent colonies around the world. Different colonies house sublines that vary substantially with respect to the frequency and severity of disease as well as the immunological characteristics of the animals (18–20). The BB rat exists in at least 24 inbred and 2 outbred lines (21). Prins *et al.* have analyzed 19 protein markers in these substrains. They observed polymorphisms in 9 markers and used them to define 7 distinct haplotypes. The data suggest that there is considerable variability, and that the genetics of each line must be considered when comparing results obtained using animals from different colonies. BB rats are often characterized according to origin by the addition of a geographic designator to the name; BB/Wor rats are from the Worcester colony, BB/E rats from Edinburgh, and so on. The importance of genetics is highlighted by the report of phlebotomy as a method for preventing BB rat diabetes (22); the protective effect was lost after the recognition that a genetic change had occurred and contaminated the observations (23). NOD mice also suffer from similar geographic variation. In some substrains the frequency of diabetes can be >90% in females and 50% in males, whereas in other substrains the frequency in both sexes can be less than 5% (18).

It is important to exercise caution when generalizing

rodent observations that may have been made in only a single location with a single substrain under local environmental influences.

#### 5.2. Environmental Factors: Diet

In addition to the genetic variation among colonies of generic “animal models” of disease, the geographic distribution of the animals introduces other variables. These include idiosyncratic exposure to diet, viruses, and other environmental factors.

The importance of diet, particularly protein that is derived from cow's milk, in the pathogenesis of human IDDM is currently a controversial subject that is under intensive study (24, 25). In part, this line of research is driven by the finding that changes in food, particularly its protein content, influence the onset of diabetes in NOD mice (26). Pregestimil, a cow milk-free infant diet, prevents diabetes in female NOD mice when given as their only protein source. In contrast, chloroform-methanol extracts of commercial mouse chow added to Pregestimil increase the incidence of diabetes (27). These observations argue that complex natural ingredients in chow are diabetogenic accelerators that are absent in semipurified diets. There are, however, additional complexities that are still more difficult to explain. Feeding NOD mice a conventional diet, but giving them access to it only every other day, decreases the incidence of IDDM (28). This last effect has not been observed in the BB rat.

The importance of dietary protein in the expression of BB rat IDDM is also clear (26). Laboratory diets including wheat gluten flour and soybean meal are associated with the maximum frequency of diabetes (29). In contrast, semisynthetic diets and diets based on hydrolyzed casein reduce the incidence of diabetes and delay the age at onset (29, 30). The mechanisms are unknown. Diets deficient in essential fatty acids also reduce the frequency of diabetes in both DP- and RT6-depleted DR rats (31). A final observation concerning diet and autoimmunity in the BB rat relates to the frequency of thyroid disease. Dietary supplementation with iodide increases the frequency of lymphocytic thyroiditis (32, 33).

The impact of diet on diabetes incidence in animal models has two implications. On the one hand, dietary protein modifications may prove to be a common thread in IDDM and its models that can be exploited to define relevant mechanisms and generate interventions. On the other, variation in diet from colony to colony (and from season to season according to the availability of raw materials) may confound the interpretation of other interventions targeted at other underlying pathogenic mechanisms.

#### 5.3 Environmental Factors: Infectious Agents

Certain viruses influence the expression of insulin-dependent diabetes in NOD mice, BB rats, and hu-



mans. In humans the temporal relationship of infection to diabetes is well documented, but the exact role of the virus in pathogenesis is unknown (34). Implicated viruses include Coxsackie B4, rubella, and mumps.

Compared with mice raised in conventional animal facilities, cesarian-derived NOD mice reared in a gnotobiotic environment become diabetic more often and at a younger age (35). Viruses documented to decrease the incidence of diabetes in NOD mice include mouse hepatitis virus, sendai, lymphocytic choriomeningitis virus (LCMV), vaccinia, lactate dehydrogenase virus, and Pichinde (7). These observations do not address the issue of retroviral infection and activation which remains an area of controversy (36).

Rederivation of the NIH resource colony of DP-BB rats into a viral antibody-free (VAF) environment has also increased the frequency of diabetes and lowered the age at onset (37). As in the NOD mouse, LCMV infection decreases the frequency of IDDM in DP-BB rats. DR-BB rats housed in VAF facilities remain free of spontaneous diabetes, but DR-BB rats infected with Kilham's rat virus (KRV) can become diabetic. Naturally occurring infection, transmitted by close contact, typically induces diabetes in  $\approx 1\text{--}2\%$  of animals (37); direct injection of KRV renders  $\approx 30\%$  of animals diabetic (38). Disease induction does not involve infection of the pancreatic  $\beta$  cells (39). Our interpretation of the data holds that KRV triggers diabetes by altering the balance between autoreactive T cells and  $\text{RT6}^+$  regulatory T cells (9).

This constellation of findings in NOD mice and BB rats provides an attractive example of the interaction of genetics and environment that is suitable for further analysis. It reemphasizes, however, the need for rigorous definition of animal status in reporting results that concern any area of pathogenesis.

#### 5.4. Environmental Factors: Housing Conditions

Increasing the ambient temperature in a colony of NOD mice reportedly lowers the frequency of IDDM (40). Not yet replicated, this report suggests the kind of poorly understood phenomena that could be affecting our interpretation of NOD mouse pathogenesis data. Higher ambient temperature was associated with lower food intake. What effect changing the temperature may have also caused with respect to metabolism, activity, and infection is difficult to say.

### 6. PREVENTION AND CURE: SEPARATING WHEAT AND CHAFF

To this point we have emphasized analogies between rodent and human IDDM and caveats that apply to the extrapolation of data sets derived from one species to another. We are next left with the need to decide how to use the information from animal models appropri-

ately. This is particularly true with respect to data that suggest ways to prevent or cure the disease in humans.

#### 6.1 Preventing NOD Diabetes

As indicated in Tables 3 and 4, the number of interventions that delay, ameliorate, or prevent diabetes in the NOD mouse is remarkably large (13, 41). There is little reason to doubt the utility of any of these interventions in the mouse; the issue is what lessons the data offer. The first lesson may be that an autoimmune process that selectively destroys pancreatic  $\beta$  cells requires the complex orchestration of the entire immune system. Interference with any of a large number of elements within that system may preclude the end result of the process: overt diabetes.

General immunosuppression is clearly effective in autoimmune diabetes as it affects all three species under discussion. It might be the final word were it not for its toxicity. The NOD data suggest that more selective interventions might be equally effective—if only we knew which were specifically pertinent to IDDM. Until we know what the earliest events in the pathogenesis are, we cannot know whether or not a given intervention is specific or nonspecific.

The data from the NOD suggest, in fact, that this model in particular is immunologically fragile. Almost anything that interferes with any element in the immune system prevents the disease. Interventions that have no documented effect on human immunity (elevated temperature, reduced caloric intake) seem to be effective. Given the refractory nature of autoimmune disease in general in humans, it is implausible that many of these interventions are relevant to the human.

Several agents that may stimulate the immune system also reduce the frequency of NOD mouse diabetes. These include, among others, lipopolysaccharide (LPS) and "irrelevant" monoclonal reagents that are directed against TCR  $\text{V}\beta$  clonotypes that are not present in NOD mice. Are these interventions in some way overcoming an inherent defect within the NOD immune system?

#### 6.2. Preventing BB Rat Diabetes

It is not as easy to prevent IDDM in BB rats as in NOD mice (see Tables 3 and 4). Interventions that prevent diabetes in the NOD mouse but not in the BB rat include nicotinamide, calorie restriction, ciaramexone, deferroxamine, and gonadectomy. Nonetheless, the list of effective interventions in the rat is still substantial (9, 10). Most agents causing generalized immunosuppression are effective, as they are in the mouse. Others appear unique to the model. Transfusion to overcome effects of lymphopenia is a particularly effective intervention in the DP-BB rat. It both restores  $\text{RT6}^+$  T cells to the circulation and prevents IDDM and thyroiditis. The therapy does not address the cause of either lym-

phopenia or diabetes, but does suggest the potential importance of peripheral suppression.

### 6.3. Which Is the Right Model for Designing Prevention Strategies?

We have raised general issues with respect to extrapolation from animals to humans. We next consider some of the specific interventions with documented effects in the various species that have led to the design and implementation of trials in children.

**Immunosuppression** Generalized immunosuppression is effective in both the BB rat and the NOD mouse. Cyclosporine is documented to be effective in the amelioration of human IDDM after its onset. There is little reason to doubt that newer immunosuppressive agents would also be effective. The crucial contemporary issue is to use the animal models to identify immunosuppressive agents that possess a better benefit to risk ratio. For the moment, no agents with such properties have been forthcoming, and no large-scale trials of generalized immunosuppression for IDDM in children are in progress.

**Parenteral Insulin.** Injections of insulin given at an early age prevent diabetes in NOD mice (42), DP-BB rats (43), and RT6-depleted DR-BB rats (44). These observations have led to suggestive favorable preliminary data in humans (45) and a large-scale human trial is underway. The BB rat data suggest that the intervention in that species is likely to be islet specific and require some degree of  $\beta$  cell involution, but detailed understanding is still lacking. Relatively substantial doses of insulin are required. In contrast, in the NOD mouse the effect may require only modest doses of insulin. If insulin injections induce tolerance (as suggested by the mouse data), the intervention may well be appropriate for humans; but if  $\beta$  cell rest is required (as suggested by the rat data), then auguries for the human trial will be less favorable. In theory, the administration of an autoantigen as a tolerizing agent is not free of the risk of disease induction.

**Oral insulin.** Oral tolerance induction by feeding peptides has gathered substantial recent attention. Favorable effects have been reported in several autoimmune diseases (46). Feeding oral insulin to NOD mice prevents diabetes, but the effects of oral insulin in BB rats have not yet been reported. Preliminary studies of oral tolerance induction in humans using insulin have been initiated on the basis of the available animal data.

**Diet.** Antibodies reactive against a peptide sequence contained within bovine serum albumin (BSA) have been observed in humans, BB rats, and NOD mice. These antibodies cross-react with an islet antigen designated p69 (47). Together with suggestive ep-

idemiological data, these findings have given rise to the theory that dietary cow's milk may contribute to the pathogenesis of IDDM in susceptible individuals across species (24, 48). The reactivity of humans with IDDM to BSA is disputed (25), but removal of dietary bovine proteins reportedly does prevent diabetes in NOD mice and DP-BB rats (48). As was the case with parenteral insulin, consistent observations made in three species have engendered a large-scale trial of dietary BSA restriction in children at risk of IDDM in Finland.

**Nicotinamide.** This agent is a precursor for nicotine adenine dinucleotide (NAD) synthesis and an inhibitor of poly(ADP-ribose) synthetase that is known to protect  $\beta$  cells from certain toxins and the effects of nitric oxide. Nicotinamide reportedly prevents IDDM in NOD mice (49). It is not effective in the BB rat (50). A large-scale intervention in children at risk is in progress despite the interspecific discordance.

## 7. CONCLUSION

We have outlined some of the extraordinary analogies that appear to exist between human IDDM and clinically, morphologically, and immunologically similar disorders in BB rats and NOD mice. These analogies will continue to engender creative experimentation and, increasingly, human clinical intervention. But we should not lose perspective. These are at some level all different diseases. At times the NOD mouse and the BB rat appear to be teaching different lessons about human IDDM. They both need to be scrutinized carefully with the "necessary caution and wisdom," called for by Renold and Shafrir (8). The outcome of the current human prevention trials based on animal model precedents will educate us considerably on the utility of this method of procedure.

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